

Properties of active nucleosomes as revealed by HMG 14 and 17 chromatography

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Received 1 December 1981; Revised 10 February 1982; Accepted 22 February 1982

SUMMARY

Nucleosomes from actively transcribed genes (active nucleosomes) contain nonhistone proteins HMG 14 and 17 and are preferentially sensitive to digestion by DNase I. Active nucleosomes isolated by chromatography on an HMG 14 and 17 glass bead affinity column were analyzed with respect to overall structure, accessory nonhistone components and modifications to the DNA and histones. The experiments lead to the following conclusions: The DNA in the active nucleosome is undermethylated compared to bulk DNA; topoisomerase I is a non-stoichiometric component of the active nucleosome fraction; the level of histone acetylation is enriched in active nucleosomes, but the extent of enrichment cannot account for HMG binding; and the two histone H3 molecules in the active nucleosome can dimerize more readily and are, therefore, probably closer together than those in the bulk of the nucleosomes. Additionally it is shown that HMG 14 and 17 prefer to bind to single- vs. double-stranded nucleic acids. The role of HMG 14 and 17 in producing a highly DNase I sensitive structure and correspondingly helping to facilitate transcription is discussed in terms of these properties.

INTRODUCTION

The most elementary subunit of eucaryotic chromosomes is the nucleosome. The structure of the nucleosome has been extensively studied, both with respect to internal structure and higher order interactions, over the past ten years using a number of different techniques (for a review see ref. (1)). It is now fairly clear that a typical nucleosome contains approximately 200 base pairs of DNA wrapped around an octamer of four histones to yield a structure having a two-fold axis of symmetry. Recently Shick *et al.* (2) delineated the spatial arrangement of the histones within the central octamer. The majority of these studies concerned themselves with total nucleosomes, 80% of which are not transcribed. This paper attempts to characterize the active nucleosomes - the 20% which are transcribed or are in a

transcribable conformation.

The properties of active nucleosomes have been studied in two fashions (see review by Mathis et al. ref. (3)). One has been to attempt to physically separate, by various fractionation methods, active from bulk chromatin and then to investigate the differences. The other has been to use various enzymes as probes in hopes that they would recognize something special about active genes.

Weintraub and Groudine (4) showed that the globin gene is preferentially sensitive to digestion by DNase I in chick erythrocyte chromatin but not in brain, fibroblast or oviduct chromatin. This sensitivity has been correlated, by reconstitution studies, with the presence of two non-histone proteins, high mobility group proteins 14 and 17 (HMG 14 and 17) which are eluted from chromatin in 0.35 M NaCl (5, 6). These proteins (see review edited by Johns (7)) are present in nuclei at a level of approximately 5% by weight compared to DNA, or at about one per ten nucleosomes. HMG proteins are highly conserved and reconstitution experiments indicated that they are not tissue or species specific. That is, HMG 14 and 17 which have been isolated from many different sources are able to sensitize to DNase I, when reconstituted on to HMG-depleted chromatin, those genes which were originally active in the cell type from which the chromatin was prepared. Thus it was concluded that besides their association with HMG 14 and 17, active nucleosomes have at least one other feature which distinguishes them from bulk nucleosomes and insures proper HMG binding and DNase I sensitization.

The interaction of HMG 14 and 17 with active chicken nucleosomes was further studied by monitoring the sensitivity of specific genes - globin, ovalbumin and RAV-O and the genes coding for nuclear RNA - to DNase I after reconstitution of total 0.4 M NaCl-depleted chromatin with HMG 14 and 17 (8). These experiments led to the following conclusions: (1) Most actively transcribed genes become sensitized to DNase I by HMG 14 and 17. (2) Either HMG 14 or 17 alone can sensitize most genes to DNase I. (3) Genes which are transcribed at different rates have about the same affinity for HMG 14 and 17. (4) HMG 14 and 17 bind stoichiometrically to actively transcribed nucleosomes. (5) HMG 14 and 17 can restore DNase I sensitivity to purified nucleosome core particles depleted of HMGs. This last observation suggests, as do reconstitution studies of Albright et al. (9) and electrophoretic studies by Sandeen et

al. (10), and Goodwin *et al.* (11) that HMG 14 and 17 do not simply substitute for histone H1, which is presumed to bind primarily to the internucleosomal linker DNA.

Obviously, the most direct approach to study active nucleosome structure would be to isolate and purify them from template active chromatin. Enrichments of this sort have been performed by digesting chromatin with nucleases which cut between nucleosomes and then separating active from bulk nucleosomes either by making use of a solubility property of active nucleosomes (12-14) or of a difference in electrophoretic properties (15, 16). A disadvantage of the solubility fractionation techniques is that they rely either on chromosomal differences relating to higher order nucleosome structure or on ongoing transcription (which in itself might be related to higher order structure). Thus, genes which are transcribed at different rates might be represented in different proportions in the active fraction. One also has to be cautious in interpreting electrophoretic active nucleosome fractionation studies since HMGs released by nuclease digestion may rebind nonspecifically to bulk nucleosomes - when the specific sites have been digested away - thereby changing their electrophoretic properties.

Recently (17) a procedure to isolate active chicken nucleosomes was described based on coupling HMG 14 and 17 to agarose or glass beads and using this "HMG column" as an active nucleosome affinity column. A preliminary characterization of the active fraction was presented. These observations indicated a direct correspondence between chromosomal regions which are capable of interacting with HMG 14 and 17 and the regions which are highly sensitive to digestion by DNase I. Since sensitivity to DNase I reflects the potential for a gene to be transcribed and not transcription per se (4), genes which are present in equal numbers but transcribed at different rates are represented equally in the HMG column active fraction. Chromosomal regions adjacent to the highly DNase I sensitive regions have also been shown to be distinguishable from the bulk of the nuclear chromatin by their DNase I sensitivity which is intermediate between expressed regions and inactive regions (18, 19). These regions do not bind specifically to the HMG column and their DNase I sensitivity could possibly be related to some higher order nucleosome structure. Here a more detailed characterization of active nucleosomes is presented and a possible model

for the function of HMG 14 and 17 in transcription is suggested.

EXPERIMENTAL PROCEDURES

Cells, chromatin and nucleosome preparation

Erythrocytes were isolated from the circulating blood of 14-16-day-old chick embryos by vein puncture or from adult Xenopus laevis by heart puncture. MSB cells (a line of chicken leukemia cells transformed by Marek's disease virus) were grown in RPMI 1640 (Gibco) supplemented with 10% calf serum. Where noted sodium butyrate was added to the medium at a concentration of 50 mM. MSB cells were labeled with carrier-free ^{32}P -orthophosphate (Amersham) at 1 mCi/ml in phosphate-free DMEM (Gibco) for 16 hrs. Nuclei were isolated by suspension in reticulocyte standard buffer (RSB) (0.01 M Tris-HCl pH 7.4, 0.01 M NaCl, 5 mM MgCl_2) containing 0.5% Nonidet P-40 (NP-40) for chicken cells and 0.05% NP-40 for Xenopus cells.

Chromatin and nucleosomes were prepared by micrococcal nuclease digestion and sucrose gradient centrifugation as described in Weisbrod and Weintraub (17). Nucleosomes were assayed by mobility on 4.5% polyacrylamide slab gels, according to the method of Albanese and Weintraub (15). All procedures were performed in the presence of 1 mM phenylmethylsulfonyl fluoride as a proteolysis inhibitor. Where noted sodium butyrate was added to 5 mM as an inhibitor of endogenous histone deacetylase.

Glycophase coupling

HMG 14 and 17, prepared and labelled as previously described (8), were covalently coupled to glycophase using a modification of the procedure of Bethell et al. (20). CDI (1:1 carbonyldiimidazole (CDI) (Koch-Light)) was recrystallized out of THF (tetrahydrofuran, distilled from lithium aluminum hydride (LiAlH_4)) and kept in vacuo at 4°C until use. 10 ml of packed glycophase (CPG/460 glycophase G (Pierce)) was thoroughly washed with dioxane (purified free of peroxides on a neutral aluminum oxide resin (Woelm type W200)). 0.4 grams of CDI was dissolved in 15 ml of dioxane and was immediately added to the glycophase. The mixture was slowly shaken for 15 minutes at room temperature. The activated beads were then washed with 100 ml of dioxane, 100 ml of 50% dioxane/50% 0.1M Naborate, pH 9.7 and finally with 100 ml of 0.1M Naborate, pH 9.7. 5 mg of HMG 14 and 17 (doped with 10^6 cpm ^3H -SP HMG-14 and 17, S.A. 1×10^5 cpm/ μg) was then added to the packed beads in

25 ml of 0.1 M Naborate, pH 9.7. The coupling was allowed to proceed for 36 hours at 4°C on a wrist-action shaker. The resin was then washed alternately 5 times with 0.1 M NaBicarbonate and 0.1 M NaAcetate (pH 4.0) each containing 0.5 M NaCl. Usually greater than 60% of the label was bound to the resin. The HMG-resin is then stored in 0.9 M NaCl; 5 mM NaPhosphate (NaP); 0.01% NaAzide, pH 7.0 at 4°C. For long time storage glycerol is added to 50%.

HMG resin binding and elution.

HMG resin was washed two times with 0.4 M NaCl; 5 mM NaP, pH 7.0 and combined with sheared, labeled, HMG-depleted-chromatin (8) at a ratio of 1 mg HMG to 0.5 mg chromatin or with depleted monomers at a ratio of 1 mg HMG to 5 mg monomers. All subsequent procedures were performed at 4°C. The mixture (approximately 10 to 20 ml) was then placed in a 6800-MW-cutoff dialysis bag (Spectropore) which was wired inside a 2.5 L roller bottle, filled with 5 mM NaP, pH 7.0, so as to prevent the bag from moving with respect to the axis of the bottle. A stir bar was also placed inside the bottle. The bottle was then placed on a roller bottle apparatus which had been modified by attachment of a commercial stir plate. The bottle was then rolled at approximately 5 rpm to keep the resin suspended during the dialysis, which was enhanced by stirring, for 16 hrs. The slurry was then packed into a 25 ml econocolumn (Biorad) and fractions were collected by washing with 5 mM NaP, pH 7.0 containing various NaCl concentrations, to yield a bound and unbound fraction.

The unbound or inactive chromatin elutes at 5 mM NaP. Non-specific binding fractions elute at 0.1 M NaCl/5 mM NaP. Bound or active chromatin elutes at 0.4 M NaCl/5 mM NaP. Extreme care should be taken to avoid either over- or under-loading the column as both will yield a bound fraction not totally representative of the active nucleosomes. The capacity of each new affinity column should be calculated empirically by varying the amount of nucleosomes loaded and hybridizing the subsequent bound and unbound DNA to specific probes. Once the capacity of column is calculated it has remained constant for up to 6 months.

DNA hybridization and dot-blotting.

DNA preparation and dot-blot analysis were performed as described in Weisbrod and Weintraub (17) except that the hybridizations were performed in the presence of 50% formamide, 10% dextran sulfate at 42°C

(21). The chicken DNA clones used to assay the various column runs are summarized by Stalder *et al.* (18). *Xenopus* DNA clones were generous gifts of Geoff Partington (B52, β -globin), Ron Reeder (Pxlr101, rRNA), Jeff Williams (E7, vitellogenin) and Donald Brown (Pxlo8, 5S RNA).

Preparation and Assay of Topoisomerase I from Active Nucleosomes

Topoisomerase I was prepared by a modification of the method of Liu and Miller (22). Active nucleosomes were diluted 2:1 with 2 x HAB (Hydroxyapatite buffer) (HAB is 2 M NaCl; 1 mM Tris-HCl, pH 7.4; 1 mM EDTA; 0.01% DTT). Hydroxyapatite (Biorad) was thinly suspended in HAB and a 2 ml column was prepared in a 5 ml disposable syringe. Approximately 20 OD₂₆₀ units of HMG-bound nucleosomes were applied to the column. Fractions were eluted stepwise with HAB, HAB containing 0.15 M NaP, and HAB containing 0.5M NaP at 65°C. The fractions containing the topoisomerase activity (eluting in HAB) were pooled and subjected to chromatography on phosphocellulose P-11 according to Liu and Miller (22). The histones subsequently were eluted in 0.15 M NaP-HAB and the DNA eluted at 0.5 M NaP-HAB.

Topoisomerase activity was measured by the relaxation of superhelical DNA. A 30 μ l reaction contained 50 mM Tris-HCl (pH 7.4), 120 mM KCl, 10 mM MgCl₂, 0.05 mM DTT, 0.5 mM EDTA, M13 RF DNA (1 μ g), and various amounts of nucleosomes or topoisomerase purified from active nucleosomes.

HPA II methylase preparation and assay.

HPA II methylase was extracted according to the procedure of Mann and Smith (23). The incorporation of methyl groups into DNA was measured as described by Quint and Cedar (24). A typical reaction mixture contained 0.1 - 5 ng DNA, 5 units HPA II methylase, 50 mM Tris-HCl (pH 7.9), 5 mM DTT and 5 μ Ci S-Adenosyl-L-(methyl-³H) methionine (78Ci/nmole) (Amersham). The reaction was incubated for one hour at 37°C and stopped by the addition of 1 ml of 10% TCA and 25 μ l of 1 mg/ml BSA. The precipitated DNA was collected on Whatman GF/A filters and counted in Aquasol-2 (New England Nuclear).

DNA Nucleotide Analysis.

5 μ g active nucleosome and unbound nucleosomal ³²P-DNA from MSB cells were digested down to 5' nucleotide monophosphates in 50 mM Tris-HCl (pH 8.0), 15 mM MgCl₂, 0.5 mM CaCl₂ with 2.5 mg/ml Pancreatic DNase I (Sigma) and 0.5 mg/ml snake venom phosphodiesterase (Worthington) for 24 hrs. at 37°C. The digests were analysed by

two-dimensional chromatography on cellulose thin-layer plates (20 x 20 cm, Merck) according to Silberklang *et al.* (25). The first dimension consisted of iso-butyric acid/NH₄OH/H₂O (66/1/33) and the second dimension consisted of 0.1 M NaPhosphate, pH 6.8/ammonium sulfate/n-propanol (100/60/2, v/w/v). Thin layer plates were exposed using a Dupont Cronex intensifying screen for 3 days using Fuji Rx film. After identifying each spot the analysis was quantitated by scraping off each spot and counting in a Beckman scintillation counter.

Histone H3 Oxidation.

Active nucleosomes and unbound nucleosomes were dialyzed into 5 mM NaP (pH 7.0), 1 mM β -mercaptoethanol. They were then dialyzed for 12 to 16 hrs. at 4°C against 10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 100 mM H₂O₂ containing various NaCl concentrations as described in the figure legend. Alternatively, the nucleosomes were dialyzed first into 5M urea, 2 M NaCl, 5 mM Tris-HCl (pH 8.0), 0.5 mM EDTA with or without β -mercaptoethanol, and then subjected to gradient dialysis down to 10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA as described by Camerini-Otero and Felsenfeld (26) except that all buffers after the urea step contained 100 mM H₂O₂. The extent of H3 dimerization was measured by SDS polyacrylamide gel electrophoresis. Histones were isolated by hydroxyapatite chromatography by a modification of the method of Simon and Felsenfeld (27). Chromatin was bound to hydroxyapatite in 0.7 M NaCl, 0.1 M NaP, pH 6.8. The column was extensively washed with this buffer to remove any traces of histones H1 and H5. The core histones were then eluted with 2M NaCl.

Gel electrophoresis.

18% polyacrylamide sodium dodecyl sulfate gels were run using the procedure of Laemmli (28) and stained with 0.1% coomassie blue. Where indicated β -mercaptoethanol was omitted from the sample buffer. Agarose gels were prepared and electrophoresed in TEA buffer (40 mM Tris-acetate, pH 8.3, 20 mM NaAcetate, 2 mM EDTA). Nucleoprotein particles were analyzed on 4½% polyacrylamide gels in ½ TEA buffer. Triton-acid-urea polyacrylamide slab gels were prepared as described by Alfacame *et al.* (29). Histones were isolated by extraction with 0.4 N H₂SO₄ followed by precipitation in 20% TCA. Gels were stained with coomassie blue, as above. Native and denaturing DNA gels were prepared according to Peacock and Dingman (30) in TBE buffer (0.09 M Tris-borate, pH 8.3; 2.5 mM EDTA). Denaturing gels contained 8 M urea.

RESULTS AND CONCLUSIONS

Isolation of Active *Xenopus laevis* Chromatin on a Chicken HMG 14 and 17 Column.

Active nucleosomes from chicken red blood cells and tissue culture cells can be purified by chromatography on a chicken HMG 14 and 17 agarose or glass bead column (17). With chicken MSB cells approximately 18% of the input material bound to the column and hybridized to greater than 40% to hnRNA, while the remaining unbound DNA hybridized to less than 5%. Weisbrod *et al.* (8) have shown that DNase I sensitivity can be restored on the integrated Rous Sarcoma Virus (RSV) genome, using HMG-depleted chromatin from quail embryo fibroblasts transformed by RSV, with chicken HMG 14 and 17. Therefore it seemed reasonable that active nucleosomes from one species could be purified on a column containing HMGs prepared from a different species.

Xenopus laevis red blood cell mononucleosomes were prepared by sucrose gradient centrifugation through 0.55 M NaCl after an extensive digestion of the nuclei with micrococcal nuclease. These nucleosomes were depleted of histone H1 and most nonhistone proteins including HMG 14 and 17, as shown by SDS gel electrophoresis. The *Xenopus* nucleosomes in 0.4 M NaCl, were mixed with a glass bead HMG column and dialyzed down to 0.01 M NaPhosphate. The HMGs on this column were prepared from adult chicken red blood cells and the binding capacity of the column was previously determined using chicken red blood cell HMG-depleted nucleosomes (see Materials and Methods). Approximately 23% of the *Xenopus* erythrocyte nucleosomes bound to the column, 5% were eluted with 0.1 M NaCl and 18% with 0.4 M NaCl. When the two major fractions were reapplied to the column both ran true and, as previously shown for chicken red blood cell nucleosomes, both contribute a small amount of material to the 0.1 M NaCl fraction. In most of the experiments presented below only one NaCl step is taken - at 0.4 M. This fraction is termed the bound fraction and the nucleosomes which do not bind comprise the unbound fraction.

In order to test whether the fractionation was specific, the DNAs from the two fractions were spotted onto and covalently linked to DE81 paper. The DNAs were then assayed by dot blot hybridization to various sequence specific probes in probe excess. Figure 1 shows the results from this analysis. 10 µg of DNA from the two fractions were spotted, in duplicate, and the filters were hybridized to a globin cDNA clone

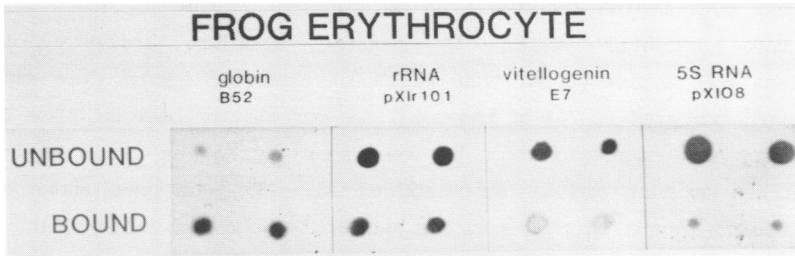


FIGURE 1. Analysis of *Xenopus* RBC bound and unbound monomers as isolated by HMG 14 and 17 chromatography by dot blot hybridization. 10 μ g each of unbound and bound DNA were spotted in duplicate onto DEB paper and hybridized to various cloned DNAs. B52 was isolated by Dr. J. Paul, E7 was isolated by Dr. J. Williams, pXl08 was isolated by Dr. D. Brown, and pXlr101 was isolated by Dr. R. Reeder.

(B52) - an expressed gene, a vitellogenin cDNA clone (E7) - a non-expressed gene, and an oocyte type 5S RNA clone (pXl08) and a ribosomal RNA (rRNA) clone (pXlr101) - two multi-copy genes, some copies of which might be expressed.

The analysis shows that the *Xenopus* globin gene is specifically bound to the chicken HMG column while the vitellogenin and 5S genes are not. The small amount of binding seen for the 5S gene is probably nonspecific but cannot be proven by these experiments. The rRNA gene binds somewhat. This could be due either to only some of the rRNA genes being active or to relatively tight, but nonspecific, binding between an altered ribosomal DNA chromatin structure and HMG 14 and 17. While it has been shown that ribosomal genes, which are transcribed by RNA polymerase I, are DNase I sensitive (31), it has not been shown that the sensitivity is due to HMG 14 and 17, as with genes transcribed by RNA polymerase II (8). Nevertheless the data clearly indicate a separation between the active globin gene and the inactive vitellogenin gene.

Active Nucleosome DNA is Depleted in 5-Methyl Cytosine

Over the last couple of years an inverse relationship has emerged between the degree of DNA methylation, as 5-methyl cytosine, and gene activation (see ref. 32 for a review). The modification predominantly occurs (90%) at the dinucleotide sequence CpG. The pattern of DNA methylation has been studied by making use of a class of restriction enzymes known as isoschizomers. The isoschizomers MSP I and HPA II have been used most often. Both recognize the same tetranucleotide CCGG, but

only MSP I cuts if the internal C is methylated. Navah-Mary and Cedar (33) have recently shown, by nearest neighbor analysis of nick-translated active genes, that 20-30% of expressed genes' CpGs are methylated, compared to 70% of the total. A correlation between DNase I sensitivity and undermethylation has been shown by Kuo *et al.* (34) in the chicken oviduct and by Weintraub *et al.* (35) who have shown a relationship, in the chicken β -globin genes, between undermethylation, DNase I sensitivity and *in vivo* and *in vitro* transcription by endogenous RNA Polymerase II.

To investigate if the active nucleosome fraction was depleted of m^5C , a nucleotide analysis was performed on ^{32}P -*in vivo* labeled chicken MSB cell DNA from bound and unbound nucleosome fractions generated by HMG column chromatography. The DNA was digested to 5' nucleotide monophosphates using DNase I and snake venom phosphodiesterase and was analyzed by two-dimensional chromatography on cellulose thin layer plates. Figure 2 shows a typical analysis. Four or five main spots are observed corresponding to pG, pA, pT, pC and pm^5C . The obvious difference one observes in comparing the pattern of spots between the

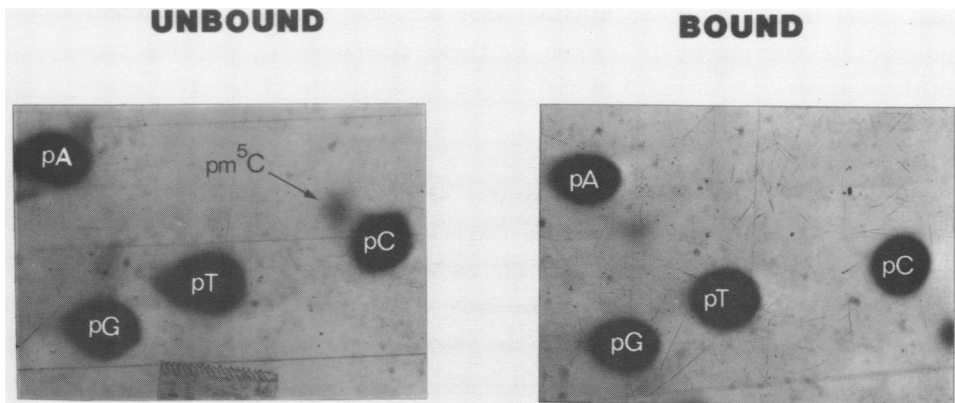


FIGURE 2. Nucleotide analysis of HMG-bound and unbound DNA. HMG column chromatography was performed on ^{32}P -HMG-depleted mononucleosomes from MSB cells. The DNA was digested down to 5' nucleotide monophosphates with DNase I and snake venom phosphodiesterase. 5 μ g of each fraction was subjected to chromatography on cellulose thin layer plates as described in Materials and Methods. The spots were identified by comparison with a parallel plate seeded with a mixture of the five pure monophosphates (not shown). The light spot next to A in the bound nucleosomes has not been conclusively identified, but might be 6-methyl adenine.

bound and unbound fractions is the lack of a spot corresponding to pm^5C in the bound fraction. The autoradiograph actually accentuates the difference because the chromatography plate had to be overexposed to see the pm^5C spot, since the ratio of pC to pm^5C is normally only about 20:1. On longer exposures the spot corresponding to pC obscures the pm^5C spot. The chromatography plates were quantitated by scraping off the spots and scintillation counting. The analysis showed roughly a 5:1 ratio of pm^5C in the unbound (bulk) DNA (approximately 4% of the total C) compared to the bound (active) DNA (approximately 0.75% of the total C).

A possible artifact of the above experiment could arise if active genes as a whole were depleted in CpG sequences. To avoid this problem, the actual amount of unmethylated CCGG sites was measured in the bound and unbound fractions using HPA II methylase and ^3H -S-adenosylmethionine (SAM) as the methyl donor. Figure 3A shows DNA dosage curves using saturating amounts of enzyme for one hour. The slope of the curve obtained by plotting the degree of methylation as a function of input DNA is a gauge of the number of methylatable sites. Figure 3B shows a time course of methylation in enzyme excess. When saturation is reached the number of available methylation sites determines the extent of incorporation. Both of these assays indicate the presence of roughly three to five times more methylatable sites in the active versus the inactive DNA fraction in agreement with the thin layer chromatography assay above.

Topoisomerase I Copurifies with Active Nucleosomes.

Previously we have shown that the inner histones in both the active and inactive nucleosome fractions are equimolar and that they comprise greater than 90% of the total protein in the two fractions. When bound and unbound nucleosomes from chicken red blood cells are overloaded on a 15% SDS polyacrylamide gel any residual histone H1 and H5, which were not removed by the high salt sucrose gradient centrifugations, are mainly found in the unbound fractions and a number of other nonhistone proteins appear in both fractions. The most prevalent nonhistone protein is a 70K protein which is found only in the bound fraction (Figure 4A). Densitometry scans of the gel indicate that the 70K protein is present at approximately 1 copy per 10 of each histone, or about one per 5 nucleosomes. The 70K protein remains with the bound nucleosomes through two more rounds of sucrose gradient centrifugation

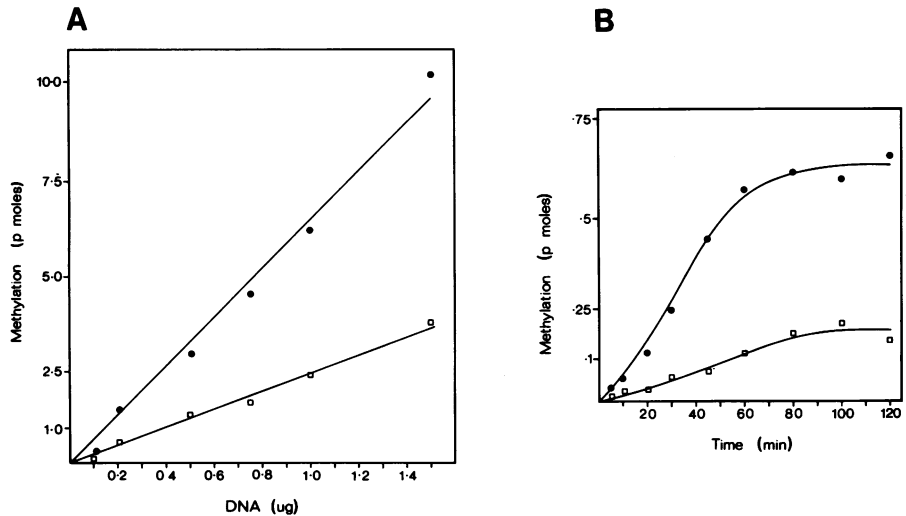


FIGURE 3. Kinetics of methylation of bound and unbound nucleosomal DNA from 14-day chick RBCs. A) DNA excess methylation analysis of bound (●) and unbound (□) fractions using 10 units HPA II methylase, 2 μ Ci 3 H-SAM (78 Ci/mMole) and increasing amounts of DNA for 2 hours at 37°C. B) Time course of methylation in enzyme and SAM excess of 0.5 μ g DNA. Symbols are as in (A).

in 0.63 M NaCl and after gel filtration on Bio-gel A 5M (not shown). Therefore it is unlikely, but still possible, that the 70K protein or an aggregate of more than one molecule of the protein, sediments at 11S (with the nucleosomes) and is partitioned by its inherent ability to bind to HMG 14 and 17.

Recently it has been shown that HMG 1 and HMG 17 could increase the rate in which topoisomerase I relaxes supercoiled DNA in an *in vitro* reaction (Javaherian *et al.* personal communication). Eucaryotic topoisomerase I (reviewed by Gellert (36)) is a ubiquitous enzyme, found in all eucaryotes and is analogous to the ω protein in *E. coli*. Various reports indicate that the enzyme is composed of a single polypeptide chain with a molecular weight between 70,000 and 120,000 daltons. Liu and Miller (22) have shown by partial peptide maps that the 70,000 dalton protein is a functional proteolytic product of the 100,000 dalton topoisomerase in HeLa cells. It thus seemed reasonable to test whether the 70K protein fractionating with the active nucleosomes was topoisomerase I.

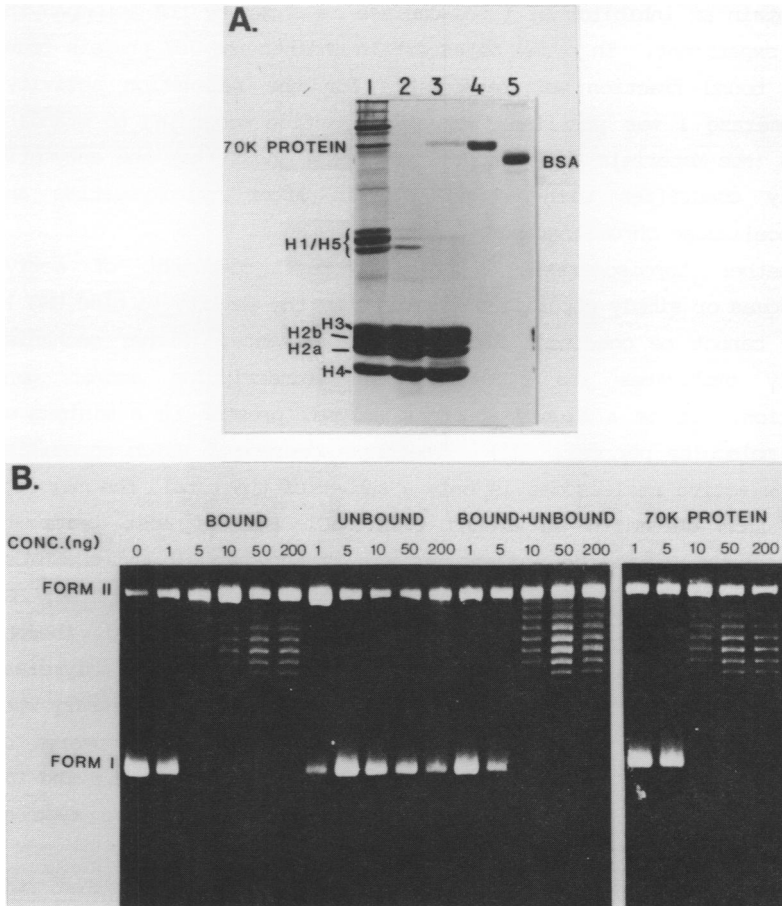


FIGURE 4. Topoisomerase I coisolates with active nucleosomes. A) 15% SDS gel of 14-day chick RBC proteins from: 1) total nuclei; 2) unbound mononucleosomes; 3) bound mononucleosomes; 4) 70K protein purified from bound mononucleosomes; and 5) BSA. Samples 2-4 were prepared by precipitation with 20% TCA and subsequent neutralization in SDS sample buffer with 1M NaBorate. The 70K protein is acetone soluble, therefore acetone washing of the precipitate must be avoided.

B) Relaxation of 0.5 μ g Form I M13 DNA with increasing amounts of bound nucleosomes, unbound nucleosomes, a 50:50 mixture of bound and unbound nucleosomes and the 70K protein purified from the bound nucleosomes. The concentrations presented for the 70K protein are in terms of the equivalent amount of nucleoprotein from which it was isolated.

Figure 4B shows that the bound but not unbound nucleosomes possess an activity which will relax supercoiled M13 DNA, under standard topoisomerase I assay conditions (22). That the unbound fraction does

not contain an inhibitor of topoisomerase is shown by the accompanying mixing experiment. In order to ascertain whether the 70K protein found in the bound fraction was responsible for the relaxation activity, topoisomerase I was purified from this fraction according to standard methods (see Materials and Methods). Figure 4 shows that the enzymatic activity copurifies with a 70K protein after hydroxyapatite and phosphocellulose chromatography.

Whether topoisomerase I is an actual component of active nucleosomes or simply copurifies with them by the ability to bind HMG 14 and 17 cannot be concluded from these experiments. Either mechanism strongly implicates its importance in transcription and/or gene activation. It is a highly abundant enzyme, present in a minimum of 70,000 molecules per cell (37). The topoisomerase I which copurifies with the active nucleosomes is only a subset of the total, the majority elutes from chromatin at around 0.3M NaCl and is lost prior to chromatography, when the nucleosomes are stripped of H1 and the HMGs. Topoisomerase I could possibly act by untwisting a transcribing DNA molecule to leave the coding strand in a planar position, thereby preventing entanglement of the nascent RNA strands as the RNA polymerase molecules negotiate the helix. This would be especially necessary when transcription (38) or transcription and replication (39) converge on opposite strands of the same piece of DNA at the same time. The DNA could not rotate in two directions at once, therefore some type of internal swivel is mandatory.

Histone Hyperacetylation is a Characteristic of Active Nucleosomes, but it is not the Reason for Binding to HMGs.

Histone acetylation has been repeatedly invoked as the mechanism whereby chromatin is activated for transcription (see review by Mathis et al. (3)) Acetylation occurs on the basic N-terminal regions or "fingers" of all four histones. There are numerous correlations between an increase in histone acetylation and increased RNA synthesis (e.g. ref. 40). Vidali et al. (41) and Sealy and Chalkley (42) have shown large enrichments in acetylated histone H4 molecules after solubilization by limited DNase I digestion. Supporting these experiments is the finding that the active fraction in the fractionation techniques of Bloom and Anderson (12), Levy et al. (13) and Gottesfeld (14) are enriched in multiacetylated forms of H4. It is important to note, though, that these studies are all correlative; none approach the

question of causation.

To test if the active fraction was enriched in acetylated histones, bound and unbound nucleosome fractions, treated in various ways with sodium butyrate, were electrophoresed on triton-acid-urea polyacrylamide gels. Candido *et al.* (43) have shown that sodium butyrate is an effective inhibitor of cellular histone deacetylases. Tissue culture cells grown with low levels (50 μ M) of sodium butyrate in the medium show greatly enhanced levels of histone acetylation. Since the treatment does not alter the acetylation reaction, the highly acetylated molecules have presumably been modified at their proper *in vivo* sites. Figure 5 I-V shows the acetylation pattern of active and bulk nucleosomes from chicken red blood cells and MSB cells prepared with or without sodium butyrate to inhibit deacetylation. In all five cases the bound fraction comprised approximately 12-20% of the input chromatin and was enriched in active sequences. The preparations handled with sodium butyrate show an increase in the level of acetylation of histones H3 and

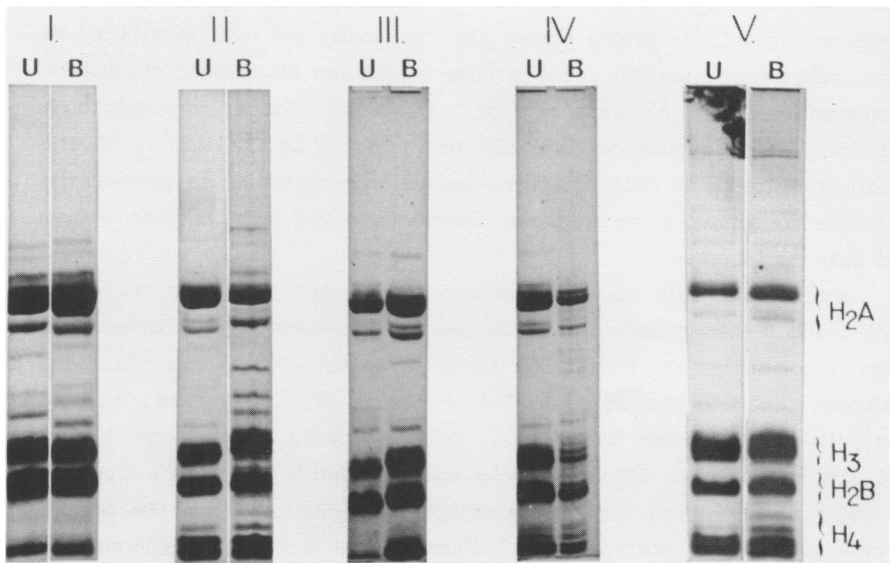


FIGURE 5. Triton-acid-urea gel analysis of bound (B) and unbound (U) proteins. I) 14-day chick RBCs; II) 14-day chick RBCs prepared with 5 mM NaButyrate in all buffers; III) MSB cells; IV) MSB cells prepared with 5 mM NaButyrate in all buffers; and V) MSB cells grown with 50 mM NaButyrate in the medium and subsequent preparation in the presence of 5 mM NaButyrate.

H4 in the bound fraction. In all five cases the degree of acetylation is nonstoichiometric and is less than one hyperacetylated histone per bound nucleosome. Therefore, histone modification alone cannot be the signal for HMGs to bind to active nucleosomes.

The Histone Octamer is Conformationally Different in Active and Bulk Nucleosomes.

The experiments above have dealt with compositional features of HMG-containing nucleosomes. Alternatively, the signal for specific HMG binding may lie in a distinct nucleoprotein conformation peculiar only to active nucleosomes. Chicken histone H3 contains a single sulfhydryl residue at position 110 of the polypeptide chain. This residue has been preserved with the exception of yeast throughout evolution (44). Image reconstruction experiments (45) have suggested that this sulfhydryl lies adjacent to the dyad axis of symmetry of the histone octamer. Prior et al. (46) have demonstrated the close proximity of the two sulfhydryl groups of the two H3 molecules in the nucleosome using pyrene labeled H3 molecules both in vivo and in vitro. Several workers have claimed to have isolated H3 dimers, which form by oxidizing the two H3 molecules yielding a disulfide bridge across the dyad axis, and have attributed to them important biological roles in the regulation of mitotic chromosome condensation and in transcription (47, 48). Since conformational differences in the histone core will be reflected in the ability of the sulfhydryl groups to oxidize, there exists an opportunity to compare the relative conformation of histones around the dyad axis between active and bulk nucleosomes.

Active and bulk nucleosomes were first dialyzed into a buffer containing β -mercaptoethanol to reduce any pre-existing H3 dimers and then into buffers of increasing ionic strength. After equilibration, hydrogen peroxide was added to 100 mM, as an oxidizing agent, and the dialysis was continued for 24 hrs. Dimer formation was assayed by SDS gel electrophoresis. Figure 6 shows the per cent H3 dimer as a function of NaCl concentration for the two fractions. Greater than 60% of the active nucleosomes are capable of dimerizing at 0.4 M NaCl, whereas the bulk nucleosome fraction dimerizes to less than 5%. At 2M NaCl as much as 20% of the bulk nucleosomes can dimerize.

Camerini-Otero and Felsenfeld (26) have shown that nucleosomes reconstituted with H3 dimers by step dialysis from 2M NaCl/5M urea, as above, are indistinguishable, by bulk structural criteria such as

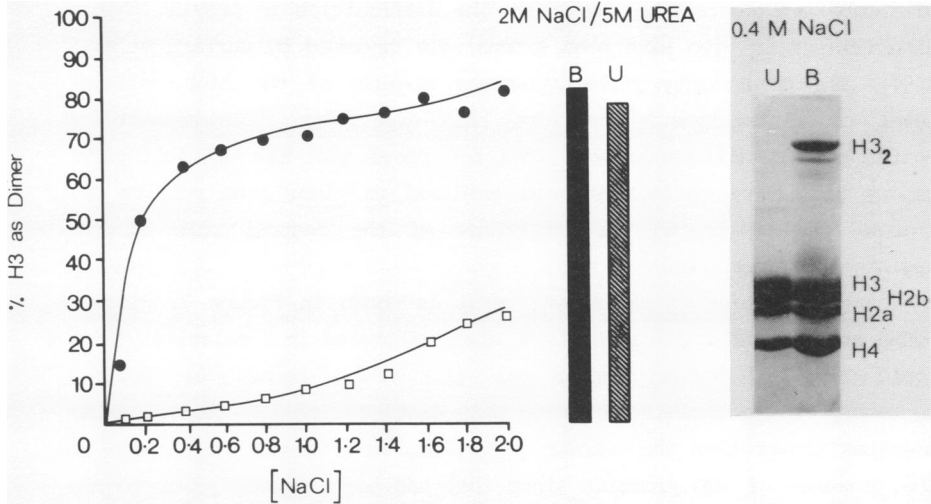


FIGURE 6. *In vitro* H3 dimer formation. 14-day chick RBC bound (●) and unbound (□) nucleosomes were dialyzed into various NaCl concentrations and then treated with 100 mM hydrogen peroxide, as an oxidizing agent. Dimerization was assayed by purifying the histones on hydroxyapatite, mixing with 2X SDS sample buffer without β -mercaptoethanol and electrophoresing in an 18% SDS polyacrylamide gel. Alternatively, as shown in the bar graph, the two fractions were dialyzed into 2M NaCl/5M urea and then reconstituted by step dialysis to 10 mM NaCl (through steps of 2.0, 1.2, 1.0, 0.8 and 0.6M NaCl) containing 10 mM Tris-HCl, pH 8.0; 10 mM EDTA and 100 mM H_2O_2 . Also shown is an SDS gel profile of unbound (U) and bound (B) oxidation in 0.4M NaCl as a representative comparison of the extent of dimerization.

susceptibility to nucleases, from native nucleosomes. A number of laboratories (see ref. (1) for review) have also reported that native core particle cysteine sulfhydryls do not react with fluorescent reporter groups but can be made to in high salt and urea. When the active and bulk nucleosomes are reconstituted in this manner, in the presence of hydrogen peroxide, there is no difference in the extent of dimerization (Figure 6). Therefore it can be concluded that there is a conformational difference between the central octomers in the two fractions which is preserved in 2M NaCl but which is abolished upon exposure to 5M urea.

The oxidation results reveal either a different or a floppier structure enabling more degrees of freedom for the H3 sulfhydryl groups in active nucleosomes and increases the probability that, *in vitro*, they

will come into contact and oxidize. The difficulties in proving that a dimer exists in vivo have been extensively reviewed by Garrard et al., (49). The uncertainty probably arises because of the high cellular level of glutathione. Therefore this experiment is a measure of conformational differences and does not prove that the H3 sulfhydryl groups in active nucleosomes are oxidized in vivo. The results do, though, add weight to the importance of the central role of the arginine-rich tetramer.

A confirmation of the above result is shown in Figure 7. Total, bound and unbound nucleosomes were electrophoresed in a relatively high ionic strength polyacrylamide gel (15). The bound, or active, nucleoprotein particle, containing only histones and DNA (and no HMGs) migrates slower than the unbound particle. This is not simply due to the presence of HMG proteins since they had been removed prior to the separation procedure. Nor is the difference a reflection of a

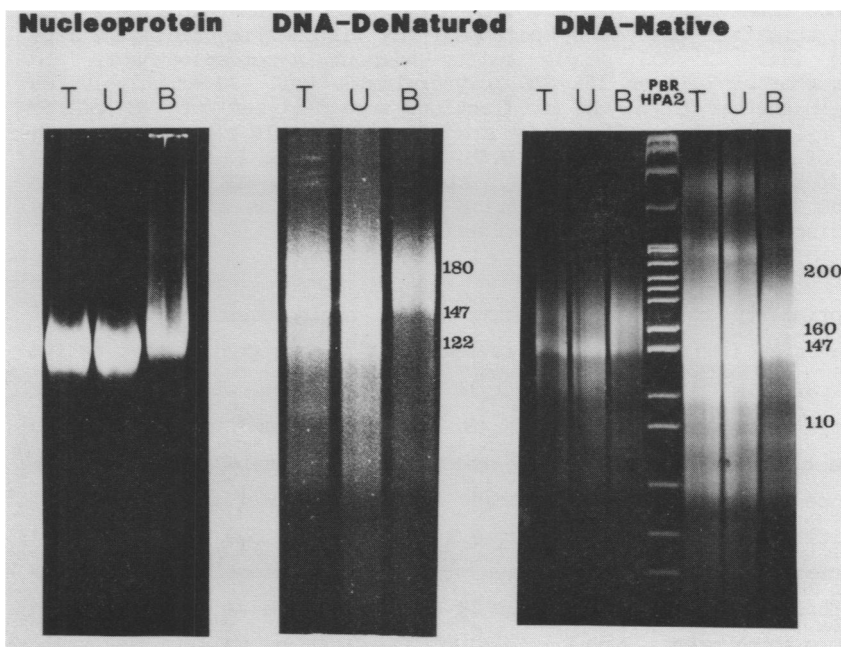


FIGURE 7. Polyacrylamide gel analysis of HMG column fractions. 14-day RBC total (T), unbound (U) and bound (B) fractions were assayed by electrophoresis on a 4 1/2% nucleoprotein gel, an 8% 8M urea denaturing DNA gel and an 8% native DNA gel as described in Materials and Methods. The DNA gels were calibrated with an HPA II digest of PBR 322.

NaCl-induced conformational change occurring during the column elution since each fraction was extensively dialyzed into 5 mM NaPhosphate prior to electrophoresis. Moreover, the difference is abolished in 0.4 M NaCl (under the same conditions which release HMGs); as shown by electrophoresing the particles in the column elution buffers (as shown previously, Weisbrod and Weintraub, (17)). Albanese and Weintraub (15) using the same gel system have shown that the monomer heterogeneity is based on charge and not on DNA length. They speculate that this could be due to differences in internal organization of the nucleosome such that fewer charged residues actually interact with the electric field and are neutralized by tightly bound counterions. This is backed up by the differential binding of spermidine by the different mononucleosome species. Moreover, they showed that the slower migrating monomer band was enriched in active sequences.

Also shown in Figure 7 are native and denaturing DNA gels of the DNA purified from the nucleoprotein particles (see Materials and Methods). Surprisingly, there appears to be less internal cleavage by micrococcal nuclease in the bound active nucleosome than in the unbound bulk nucleosomes. Additionally, the discrete stops at 146 and 160 base pairs seen in total nucleosomes are less pronounced in the bound fraction. While this could suggest possible ways in which HMG 14 and 17 bind to the nucleosome or other more subtle change in nucleosome structure, the differences are not stoichiometric and are, consequently, not responsible for HMG binding. In fact, HMG column binding does not depend on DNA length, since the separation still works with nucleosomes which have been trimmed to 145 base pairs with exonuclease III (unpublished observation).

HMGs Bind to Single-Stranded DNA Better than to Double-Stranded DNA.

The characterization of active nucleosomes discussed so far in this paper has not dealt with the question of how the HMGs confer DNase I sensitivity and what role they might play in transcription. A clue to how this interaction might take place comes from binding studies of HMG 14 and 17 to different nucleic acids. Figure 8 shows elution profiles of ^3H Form I and Form II SV40 DNA, ^{32}P -single-stranded M13 DNA and ^3H -PBR 322 cRNA on an HMG column. Notice that single-stranded nucleic acids or ones which have some single-stranded regions (i.e. supercoiled SV40) bind to the column more tightly than double-stranded DNA with respect to the NaCl concentration necessary for elution. Since rather

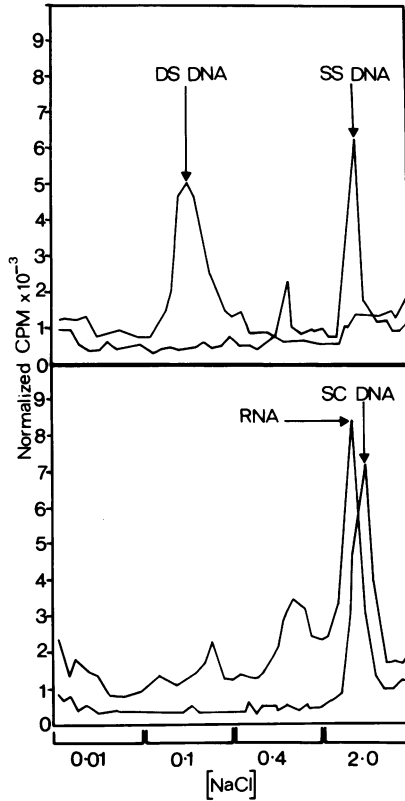


FIGURE 8. Binding of various nucleic acids to immobilized HMG 14 and 17. Samples were dialyzed onto an HMG column, in column excess, from 0.4M NaCl down to 0.01M NaCl. They were then step eluted with 0.01, 0.1, 0.4 and 2M NaCl. DS DNA, Form III ³H-SV40 double-stranded DNA; SS DNA, ³²P-M13 single-stranded DNA; RNA, ³H-PBR crRNA; and SC DNA, supercoiled Form I ³H-SV40 DNA.

large NaCl steps were taken nothing can be concluded with respect to the relative affinities of the different fractions eluting at 2M NaCl. This result is in agreement with recent data of Isackson and Reeck (50) using the opposite approach - chromatography of HMGs on single- and double-stranded DNA columns. Together with the known affinities of DNA for active and bulk nucleosomes (8, 10) the following hierarchy of salt-sensitivity of binding can be concluded: Single-stranded DNA (or RNA) > active nucleosomes > double-stranded DNA > bulk nucleosomes.

DISCUSSION

It is now clear that transcribed DNA sequences are packaged into a nucleosome or nucleosome-like structure, as is the rest of the genome. Active nucleosomes are distinguished from bulk nucleosomes by their high sensitivity to digestion by DNase I. DNase I sensitivity has subsequently been used as a handle to study their structure. In vertebrates, the sensitivity has been shown to be due to HMG 14 and 17 interacting with some alteration of the basic nucleosome structure. This interaction is responsible only for the highest level of DNase I sensitivity, characteristic of the coding, but not the flanking regions of active genes (17). The modified nucleosome conformation and composition are, presumably, needed to facilitate transcription.

Active nucleosomes can be isolated from total NaCl-depleted nucleosomes by HMG 14 and 17 affinity chromatography. Sandeen *et al.* (10) and Albright *et al.* (9) have shown that all nucleosomes actually have the ability to bind HMG 14 and 17. To insure optimal separation the ratio of active nucleosomes to functionally bound HMGs has to be greater than or equal to one. Therefore, only the relative affinities for HMGs of active and bulk nucleosomes are distinguished by the HMG column. The high affinity for HMGs of active nucleosomes can be due either to a difference in histone and/or DNA composition or to a difference in nucleoprotein conformation. The experiments presented here result in a characterization of active nucleosomes, but it is difficult to distinguish between those features which are responsible for HMG binding and those which are a consequence of transcription or replication of active genes.

Hyperacetylated histones and topoisomerase I are found in a minority of active nucleosomes. Therefore, they are probably accessory characteristics of active genes and are not necessary for the formation of the basic active nucleosome structure. Perhaps, then, they play a role in controlling rates of transcription or act in conjunction with HMGs to destabilize the nucleosome as a prelude to transcription.

The two characteristics which are common to most (or perhaps all) active nucleosomes in chickens are undermethylation and an altered histone octamer conformation. It is tempting to suggest a cause and effect relationship between these two phenomena with respect to nucleosome assembly. That is, during replication, the DNA polymerase machinery will recognize a stretch of undermethylated DNA as a gene to

be transcriptionally active. When DNA is packaged into a nucleosome, the arginine-rich histone tetramer is assembled first (51). Therefore, once the initial recognition event occurs, the active histone conformation - as evidenced by the altered positioning of histone H3 - can be initiated very close to the replication fork; prior to the completion of the octamer with histones H2a and H2b. It is not known when the HMG proteins become associated with active nucleosomes, but Weintraub (52) has shown that the DNase I sensitive structure is established very soon after replication. Presumably, HMG 14 and 17 are, then, assembled soon after the histone octamer is complete.

One of the many possible models for the behavior of active nucleosomes during transcription can be weighed with respect to the data presented above. The nucleosome could be in a dynamic equilibrium between being stably bound to duplex DNA, not interacting with RNA polymerase (the ground state), to being transiently bound to the non-coding strand (the excited state) as the polymerase copies the coding strand. The active octamer of histones need not move to the non-coding strand as long as enough of the coding strand is free of histone contacts in order to relieve any topological barriers to polymerase movement. As the polymerase reads through, the DNA will begin to reanneal, thereby enabling the nucleosome (either in parts or all together) to fall back to the energetically more favorable ground state. HMG 14 and 17 cannot melt double-stranded DNA (10) but do possess a preferential affinity for single-stranded DNA. Their function in transcription, then, might be to impose a polarity on the otherwise symmetrical nucleosome and, thereby, facilitate movement to the noncoding strand. There are two major assumptions in this model. One is that the nucleosome can stably interact with single-stranded DNA. Palter et al. (53) have shown that the histone octamer can be transiently bound (intact) to single-stranded DNA but not to RNA. Additionally, Dunn and Griffith (54) have shown that an RNA/DNA duplex cannot be reconstituted with histones into a nucleoprotein complex by NaCl gradients. Therefore, the anti-template preference of the nucleosome in this model is not unreasonable. The second assumption is that the histone octamer stays together (as it does in replication, Leffak et al. (55)) during transcription. This could be facilitated by transient histone H3 disulfide bridges between the two heterotypic histone tetramers.

The position of the HMGs in the nucleosome is not known. The model presupposes that they are assembled in such a fashion so that they are oriented towards the non-coding strand or that they will naturally orient themselves (after assembly) during transcription to the strand not being used by RNA polymerase. Bakeyev et al. (56) have characterized a subnucleosomal particle (SN₃) which contains histone H4, 27 base pairs of DNA and HMG 14 or 17. Histone H4, which is positioned down the dyad axis of symmetry from the possible H3 dimer, has also been shown to preferentially crosslink to HMG 14 and 17 (M. Leffak, personal communication). Moreover, trypsin digestion of the N-terminal regions of the histones abolishes the ability of active nucleosomes to bind to HMGs (Weisbrod and Weintraub, unpublished observations) and abolishes the electrophoretic heterogeneity of total nucleosomes (15). These results are in accordance with earlier experiments of Weintraub and Groudine (4) where it was shown that after (but not before) trypsin digestion, active mononucleosomes become preferentially sensitive to digestion by micrococcal nuclease. Thus it is likely that the HMGs are interacting with the basic histone fingers, (perhaps exclusively H4) probably through their acidic moieties. Conversely, two different 2-dimensional electrophoretic nucleosome fractionation systems (10, 16) have shown that one or two HMG molecules can be bound per nucleosome depending on the length of the internucleosomal linker DNA - predicting that the HMGs may also be interacting with DNA through their basic moieties. Taken together these experiments suggest that HMG 14 and 17 bind to the nucleosome core and cover, or interact with the internucleosomal linker DNA.

The experiments reported here deal with the structure and function of active nucleosomes. Temporally antecedent to this is the problem of establishment - that is, how the cell knows how to package some DNA into an active nucleosome conformation and some into an inactive one. One aspect of establishment is propagation. Alberts et al. (57) have subdivided propagation into two types. The first concerns itself with propagation of the active structure down the length of the transcription unit. This could occur in nucleosome-length jumps initiated either by undermethylation or by sequence-specific interactions of histone octamers and DNA; or in larger jumps e.g. the size of a replicon. The second type of propagation is from mother to daughter cell. Seidman et al. (58) have shown that the histone octamer segregates asymmetrically

during cell division; the intact octamer preferentially going to the leading strand, which is also the coding strand. An intriguing possibility is that, besides giving the nucleosome polarity for transcription, HMG 14 and 17 also function in replication by helping to insure the inheritance of a stable phenotype. This can be investigated more fully once the segregation pattern of HMG 14 and 17 is known.

ACKNOWLEDGEMENTS

I would like to thank Dr. Harold Weintraub in whose lab this work was initiated and Dr. John Gurdon for support and space for the bulk of the work. I am grateful to Dr. Leroy Liu for communicating results on topoisomerase I experiments prior to publication and to Dr. Aaron Klug for many helpful discussions. I am indebted to Drs. Graham Goodwin, Richard Harland, Ron Lasky, Aaron Klug, Kevin Struhl and Harold Weintraub for helpful comments and suggestions on the manuscript. Dr. Richard Harland is also thanked for preparations of HPA II methylase. The help of Susan Whytock and Barbara Rodbard in preparing the figures and the manuscript is greatly appreciated. This work was partially supported by American Cancer Society Postdoctoral Fellowship No. PF 1750.

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